PROTOCOL

Protocol for GLP Single Tube Method for Measuring Disinfectant Efficacy against Biofilm Grown in the CDC Biofilm Reactor
Protocol - P2109

Test Microorganism(s)
Pseudomonas aeruginosa ATCC 15442

Product Identity
Test Substance: Sterilex Ultra Disinfectont Cleoner Solution 1
Lots: RS1-188A, RS1-188B, RS1-189A

Test Substance Sterilex Ultra Activator Solution
Lots: RS1-189B, RS1-189C, RS1-190A

Data Requirement
U.S. EPA 40 CFR Port 158
U.S. EPA OCSPP 810.2200

Study Sponsor
Sterilex Corporation
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Performing Laboratory
Microchem Laboratory
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Protocol Number
P2109

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Date
20MAR2018

PROTOCOL (cont.)

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I. Introduction

This document details the materials and procedure for evaluating the efficacy of liquid disinfectants using the ASTM E2871-13 Standard Test Method for Evaluating Disinfectant Efficacy against Biofilms Grown in CDC Reactor using the Single Tube Method in accordance with Good Laboratory Practice Standards (GLPS) stipulated by 40 CFR 160. Modifications to the official method have been incorporated to account for recommendations outlined in EPA BEAD SOP MB-20. This document also explains the terms and conditions of testing.

II. Purpose

The purpose of this study is to document the efficacy of the test substance against the test system (microorganism) under the parameters specified in this protocol.

III. Justification for the Selection of Test System (Microorganism)

The test microorganism listed on page 1 of this protocol is the microorganism designated for use in the test method ASTM E2871-13 as well as designated for testing per EPA BEAD SOP MB-20.

IV. Terms and Conditions

Studies by Microchem Laboratory are conducted in accordance with general terms and conditions posted on www.MicrochemLab.com/terms

Prior to study initiation, Microchem Laboratory must receive the approved and signed protocol, test substance and payment. Changes to the signed, approved protocol will require amendment and may incur additional fees. Cancellation of the study any time after the protocol has been signed will result in a cancellation fee of up to 100% of the total study cost, to be determined by laboratory management at its sole discretion.

Microchem Laboratory may repeat studies, free of charge, in the event of unintended protocol non-conformance, if the non-conformance is determined by the Study Director to have affected the study outcome. If the neutralization system specified for a study is not adequate, the study will be deemed "inconclusive" and the Study Sponsor will be responsible for the cost of the study. In addition, the Study Sponsor is responsible for the cost of all studies performed to confirm the outcome of a previous study and for ensuring that the study will meet their regulatory objectives.

The Study Sponsor must obtain written consent from Microchem Laboratory to use or publish its protocols, study reports (or parts thereof), logo or employee names for marketing purposes.

Test substance characterization as to content, stability, etc., is the responsibility of the Study Sponsor. The test substance shall be characterized by the sponsor prior to the completion of this study.

PROTOCOL (cont.)

V. Test Substance Identification, Characterization, ond Handling

All test substances used to substantiate antimicrobial efficacy claims will be manufactured or otherwise tested at the lower certified limit (LCL).

Test Substance Name — Sterilex Ultra Disinfectant Cleaner Solution 1
Lot Number(s) — RS1-188A
Active Ingredient & Concentration — To be noted in final report
Manufacture Date — 07JUN2017
Expiration Date —07JUN2018

Test Substance Name — Sterilex Ultra Disinfectant Cleaner Solution 1
Lot Number(s) — RS1-188B
Active Ingredient & Concentration — To be noted in final report
Manufacture Date — 07JUN2017
Expiration Date — 07JUN2018

Test Substance Name — Sterilex Ultra Disinfectant Cleaner Solution 1
Lot Number(s) — RS1-189A
Active Ingredient & Concentration — To be noted in final report
Manufacture Date — 07JUN2017
Expiration Date — 07JUN2018

Test Substance Name — Sterilex Ultra Activator Solution
Lat Number(s) — RS1-189B
Active Ingredient & Concentration — N/A
Manufacture Date — 07JUN2017
Expiration Date —07JUN2019

Test Substance Name — Sterilex Ultra Activator Solution
Lot Number(s) — RS1-189C
Active Ingredient & Concentration — N/A
Manufacture Date — 07JUN2017
Expiration Date — 07JUN2019

Test Substance Name — Sterilex Ultra Activator Solution
Lot Number(s) — RS1-190A
Active Ingredient & Concentration — N/A
Manufacture Date — 07JUN2017
Expiration Date — 07JUN2019

PROTOCOL (cont.)

Special Handling Requirements — None

Test substance characterization as to content, stability, etc., (40 CFR, Part 160, and Sub part F [160.105]) is the responsibility of the Study Sponsor. The test substance shall be characterized by the Sponsor prior to the completion of this study.

Test substances and devices are handled as follows:

- The test substance is stored at ambient (room) temperature under fluorescent lighting or in a cabinet.
- The test substance is shaken or otherwise mixed well immediately prior to use (if applicable).
- The test substance is handled safely in accordance with the chemical risks it may pose, stated in the MSDS or by the Study Sponsor during the course of pre-study communication.

VI. Study Parameters, Incorporated by Reference

Number of Tests Comprising the Study — **3 (1 Test per Test Substance Lot per Test Microorganism per day)**
Carrier Type — **Sterile borosilicate glass disks (carriers)**
Number of Carriers per Test Substance Lot — **5**
Number of Carriers per Control Substance — **3**
Test Substance Form — **Dilution Required**
(1:1:2). 1 part Sterilex Ultra Disinfectant Cleaner Solution 1 + 1 part Sterilex Ultra Activator Solution + 2 parts Diluent
Test Substance Diluent — **400 ppm ± 10 ppm AOAC Hard Water**
Test Temperature — **21 ± 2°C**
Contact Time — **9 minutes 45 seconds ± 5 seconds**
Neutralization Broth — **2X Dey/Engley Broth supplemented to contain 5.0% Tween 80 and 5.0% catalase**

Proposed Experimental Start Date: **23MAR2018**
Proposed Experimental Termination Date: **23APR2018**

VII. Test System (Microorganism

Pseudomonas aeruginosa ATCC 15442

PROTOCOL (cont.)

VIII. Materials

- Pure culture of the test system (microorganism).
- Sufficient quantity of growth media (sterile Tryptic soy broth (TSB) made to appropriate concentrations per the method).
- Sufficient quantity of micropipettes and appropriately sized sterile micropipette tips.
- Ultrasonic water bath
- Peristaltic pump
- Magnetic stir plate
- Silicone tube of appropriate size
- Glass flow break
- Clamp stand and clamp
- CDC Biofilm reactor and components
- Sufficient quantity sterile borosilicate glass carriers (~1.27 cm diameter and ~3.0 mm thick)
- Carboys of appropriate size to hold >10 liters of growth media
- Sufficient volume of dilution media (Phosphate buffered saline, PBS) in appropriate volumes
- Sufficient volume of neutralization broth media in apprapriate valumes.
- Vortex mixer
- 50 mL or 250 mL canical tubes
- 0.45 um polyethersulfone (PES) filter membrane
- Filter manifold
- Splash guards
- Sufficient quantity of sterile petri dishes.
- Sufficient number and volume af sterile Petri dishes and sterile Tryptic Soy Agar (TSA), R2A agar, or other appropriate grawth agar for enumeratian af diluted microbial suspensions.
- Bunsen burner, microbiological incinerotor, or micro-torch
- Automatic pipettar (Pipet-Aid ar similar) and various sizes of sterile serological pipets.
- Thermometer (far submersian in an equilibrated test tube to indicate the temperature of the test substance during the test).
- Incubator capable af sustaining temperatures of 36±1°C.
- Forceps.
- Appropriate volume of 95% ethanol.
- Sufficient number of test tube racks.
- Certified satellite clock.
- Certified digital timer.

PROTOCOL (cont.)

IX. Procedure

Preparation of AOAC synthetic hard water solution

- From each 1000 mL of sterile deionized (DI) water (as measured by 1L volumetric flask), a volume equal to the total volume of AOAC hard water reagents added in the steps below is removed by serological pipette. For example, if 4 mL of solution "1" and 4 mL of solution "2" are to be added, then 8 mL of sterile water is removed.
- The concentration in PPM of hard water to be made is divided by 100. That is the volume, in mL, of AOAC hard water solution "1" will be needed to make 1000 mL of hard water.
- Based on the calculation above, an appropriate volume of AOAC solution "1" is added to the sterile water, and mixed.
- The appropriate volume of solution "2" is then added and mixed.
- An appropriate volume of the synthetic hard water is removed and titrated. If necessary, the solution may be diluted with sterile water or augmented with parts of solution "1" and "2" to achieve the study sponsor requested hard water level. In any case, the hard water concentration of the final solution is to be determined by titration and recorded.

Preparation of Subculture/Neutralization Media

- Before the test begins, the subculture/neutralization media is prepared in bulk and steam sterilized prior to use.

Preparation of Test Substance

- Test substance is prepared by dilution.
  - (1:1:2) by the addition of 1 part of Sterilex Ultra Disinfectant Cleaner Solution 1 to 1 part of Sterilex Ultra Activator Solution to 2 parts of AOAC Synthetic Hard Water.
    - The following dilution ratios are used to generate a 1:1:2 dilution of the test substance Sterilex Ultra Disinfectant Cleaner Solution 1 at the LCL:
      - For Lot: RS1-188A: 6.0 mL of disinfectant solution, 6.0 mL of activator solution, 12.357 mL of hard water diluent.
      - For Lot: RS1-188B: 6.0 mL of disinfectant solution, 6.0 mL of activator solution, 12.263 mL of hard water diluent.
      - For Lot: RS1-189A: 6.0 mL of disinfectant solution, 6.0 mL of activator solution, 12.304 mL of hard water diluent.
- Test substance is used within 3 hours of preparation.

Preparation of Test Vessels

- Test vessels are prepared by removing the lids and placing splashguards, flared piece up, into the vessel.
  - Care is taken to ensure the splashguard sits at the straight/conical interface of the tube.

PROTOCOL (cont.)

- Vessels are covered with foil and autaclave sterilized.
  - Tubes cantaining splashguards are anly required far reactian tubes with carriers treated with test substances.

Preparatian af carriers

- Carriers with visible damage to surface topography are discarded.
- Priar ta, ar after use in testing, carriers are submerged in DI water and autaclave sterilized.
- Glass carriers are sonicated in plastic 50 mL canical tubes for 5 minutes in a 1:100 dilution of detergent and tap water.
- Carriers are rinsed with reagent grade water and sonicated for approximately 1 minute in reagent grade water. carriers are rinsed and sonicated until no soap remains. carriers are handled with kim-wipes or delicate task wipes and are stored later use.

Preparation and Sterilization of the CDC Bioreactor

- All segments of the CDC bioreactor are disassembled, sterilized, and cleaned (as appropriate) prior to use.
- The reactor top is inverted and the baffled stir bar is placed onto glass rod pasitianed in the center of the reactor top.
- The assembled reactor top is carefully placed into the reactor beaker.
- The bacterial air vent are connected by fitting the vent to a small section of appropriately sized tubing and attaching it to one of the rigid tubes on the reactor top.
- Cleaned and screened carriers are placed into each hole in the reactor rods, leaving the top of the carrier flush with the inside rod surface. carriers are secured by tightening the set screw.
- Rods are placed loosely into reactor top (not yet fitted into notches).
- The end of the injection ports and any extra openings on the completed reactor are covered with aluminum foil or plastic caps.
- The completed reactor is autoclave sterilized prior to use.

Preparation of Test Culture

- For *P. aeruginoso*, a culture of the test microorganism is created from the microbial library stock plate. A single colony of test microorganisms is harvested from the stock plate and added to a tube containing 10 mL of TSB (0.3 g/L). The tube is vortex mixed and incubated at 36±1°C for 24±2 hours.

Growth of Biofilm in CDC Bioreactor – Batch Phase

- The overflow line is clamped. Aseptically add 500 mL of the batch culture medium to the cooled reactor (e.g., carefully remove one rod, pour the medium into the reactor through the rod opening, and re-insert the rod).
  - For *P. aeruginosa*, the batch culture medium is 0.3 g/L TSB.
- The rod alignment pins are secured into the reactor top notches. The prepared reactor is placed on a stir plate.

PROTOCOL (cont.)

- The flow break is clamped in on upright position.
- The 10 mL tube of culture is vortex mixed. 1 mL of the culture is used to inoculate the reactor through one of the available rigid stainless steel tubes in the reactor top.
- The magnetic stir plate is turned on.
- For *P. aeruginoso*, the rotational speed of the baffle is 125 ± 5 rpm. Incubate the reactor system in batch phase at room temperoture far 24±2 haurs. Pre-heating the 500 mL batch phose growth medium is not required.

Preparation of Continuously Stirred Tonk Reactor Medium

- The *P. aeruginoso* biofilm is grown at room temperoture during continuously stirred tank reoctor phase.
- The growth medium is prepored to achieve a final growth medium cancentrotion of 0.1 g/L TSB in the carboy.
- The growth medium tubing line is aseptically cannected to the carboy containing the continuously stirred tonk reactor mode growth medium.

Growth of Biofilm in CDC Reactor – Continuously Stirred Tank Reactor Mode

- A cantinuous flaw af growth medium is pumped into the reactor ta achieve a 30 ± 2 minutes residence time based on the reactor's operating volume. Connect the end of the reactor drain to the waste carboy and remove the clamp.
  - Flow rate is calculated by dividing the reactor volume by the residence time (30 ± 2 minutes). The reactor volume (with the 8 rods and baffled stir bar in aperation) is approximately 325 mL.
- For *P. aeruginosa*, aperate the reactor in CSTR mode for 24±2 hours at room temperature.

Exposure of Carriers to Test Substance

- The growth medium flow and baffle stir bar are turned off.
- A randomly selected rad containing carriers with biofilm is aseptically removed from the CDC Biofilm Reactar by firmly pulling it straight up out of the reactor.
  - Carriers are used withing 30 minutes of removal from the bioreactor.
- The carriers are rinsed to remove planktanic cells.
  - The rod is oriented in a vertical position directly over a 50 mL canical tube containing 30 mL PBS.
  - The rod is immersed with a cantinuous matian into the PBS with minimal to no splashing, then immediately removed.
  - A new 50 mL conical tube with 30 mL PBS is used for each rod.
- The rod is held with one of the randomly selected carriers centered over an empty, sterile vessel.
  - Examples of vessels are 50 mL or 250 mL conical tube containing splashguards.
- During carrier deposition, the rod shauld nat make contact with the tube ar splashguard far treated ar control samples.

PROTOCOL (cont.)

- The set screw is loasened using a flame sterilized Allen wrench and the corrier is allawed to drop directly to the bottom of the tube.
  - If the carrier does not freely drop, the center of the corrier is pressed with the Allen wrench used to loosen the set screw.
- Five tubes, each with one carrier, are prepared for treatment with the test substance.
- Three tubes, each with one corrier, are prepared for treatment with the control substance.
- After the corriers are deposited, the sploshguards are aseptically removed from each tube using sterile forceps.
- Corriers to be treated with test substance are treated first.
- 4.0 mL of the prepared test substance or control substance (PBS) are applied down the side of the tubes containing the corriers. Corriers are treated at appropriate time intervals to ensure careful and aseptic handling.
  - Direct contact with the carrier should be avoided during applicotion. Corriers should be completely covered with the test or control substance.
- The tubes containing test or control substance are gently swirled to ensure no air bubbles are trapped beneath the corrier and to fully expose the biofilm to the liquid.
- Tubes are kept at the contact temperature for the duration of the contact time.
- At the end of the contact time, 76.0 mL of the appropriate neutrolizer is added to each tube. Tubes are briefly vortex mixed after initial neutralization.
- Tubes are vortex mixed on the highest setting for 30±5 seconds.
- The tubes are suspended in a sonicating water bath and sonicated for 30±5 seconds.
  - Each liquid level in the tubes should be even with the liquid level in the bath. The tubes are not allowed to touch the bottom or sides of the ultrasonic water bath.
- Tubes are vortex mixed, a second time, on the highest setting for 30±5 seconds.
- The tubes are suspended in a sonicating water bath and sonicated for 30±5 seconds a second time.
  - Each liquid level in the tubes should be even with the liquid level in the bath. The tubes are not allowed to touch the bottom or sides of the ultrasonic water bath
- Tubes are vortex mixed, a third time, on the highest setting for 30±5 seconds.
- Tubes containing the carrier are the 10^0 dilution. Each 10^0 dilution is serially diluted (1:10) for treated and control carriers in 9.0 mL blanks of PBS.
  - A minimum of 10 mL from the 10^0 dilution and the entire contents of the 10^{-1} dilution tube (10 mL) are filtered through a 0.45 μm PES filter membrane for treated carriers
- For filtration, membrane filters are pre-wet with ~20 mL PBS. The entire contents of the either the 10^0 tube or respective dilution tube are passed through a filter. The filtered tube is rinsed with ~10 mL PBS and the rinsate is filtered. The sides of the filter funnel are rinsed with additional PBS and membrane filter is plated on R2A agar.
  - For controls, spread or pour plate appropriate dilutions to achieve colony counts in the range of 30-300 colony forming units (CFU) per plate (e.g., 10^{-4} and 10^{-5}).
- All plates are incubated for 48 ± 4 hours at 36±1°C.

PROTOCOL (cont.)

Neutralization Control

- For *P. aeruginoso*, o 24±2 hour culture is initiated in 10 mL of 30 g/L tryptic soy broth. The culture is incubated at 36°C±1°C.
- Neutrolizotion Confirmation Treotment (NCT). At timed intervols, 4.0 mL of test substance is odded to eoch of 3 tubes contoining 76 mL neutrolizer. Briefly mix, within 10 seconds, odd 0.1 mL of the test orgonism diluted to 10^5 CFU/mL, ond vortex to mix thoroughly. Additionol dilutions of the test microorgonism moy be prepored and verified.
- Neutrolizer Toxicity Treotment (NTT). At timed intervols, 0.1 mL of the test orgonism diluted to 10^5 CFU/mL is odded to eoch of 3 tubes containing 80 mL neutralizer ond vortex to mix thoroughly. Additionol dilutions of the test microorganism may be prepored ond verified.
- Test Culture Titer (TCT). At timed intervols, add 0.1 mL of test orgonism diluted to 10^5 CFU/mL is odded to each of 3 tubes containing 80 mL dilution buffer and vortex to mix thoroughly. Additional dilutions of the test microorganism may be prepored and verified.
- Hold all treatments at room temperature for the contact time.
- After the contact time, vortex each tube thoroughly and prepare one 10-fold dilution in 9.0 mL dilution buffer.
- Briefly vortex the dilution tube prior to plating; initiate plating within 30 minutes of making dilutions. Plate 0.1 mL aliquots from each tube in duplicate on R2A plates using spread plating. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubatian.

Media Sterility Control

- An aliquot of PBS is added to sterile growth medium and incubated alongside enumeration plates to verify sterility at the time of test.
- An aliquot of the test substance diluent is added to sterile growth medium and incubated alongside enumeration plates to verify sterility at the time of test.
- A plate containing only growth medium used in this study is incubated alongside enumeration plates to verify sterility at the time of test.

Incubation of Tubes and Enumeration and Control Plates

- Plates are incubated at 36 ± 1°C for 48 ± 4 hours.

PROTOCOL (cont.)

X. Calculations

- Colony counts of >200 CFU/filter are recorded as TNTC. For treated carrier calculations, values of 200 will be substituted for TNTC and are scaled up.

$$\frac{\frac{(\text{Average CFU for } 10^{-X}) + (\text{Average CFU for } 10^{-Y})}{10^{-X} + 10^{-Y}}}{Z} = \text{CFU/mL}$$

Where X and Y are dilutions plated and Z is is the volume plated.

[(CFU/mL) * A] = CFU/Carrier
Where A is the volume of neutralizer and test substance in the tube.

Log_{10} density per carrier (treated or control) = log_{10}(CFU/Carrier)

Log_{10} reduction = mean log_{10} control carriers – mean log_{10} treated carriers
When this is no recovery for treated carriers and the entire 10^0 tube is filtered, the log reduction is noted as greater than the mean control counts.

XI. Success Criteria

- The experimental success (controls) criteria follow:
  - The test microorganism must demonstrate a mean log density of between 8.0 and 9.5 with each carrier exhibiting a log density between 8.0 and 9.5.
  - The neutralization control inoculum demonstrates <300 CFU/plate.
    - The recovered CFU in the Neutralizer Toxicity Treatment (NTT) is within 50% of the Test Culture Titer (TCT).
    - The recovered CFU in the Neutralizer Confirmation Treatment (NCT) is within 50% of the Test Culture Titer (TCT).
  - The media sterility controls are negative for growth.

- The EPA performance criterion for disinfection follows:
  - A minimum of 6 log reduction is observed in the viable bacteria on treated carriers compared to control carriers.

XII. Reporting

- Results are reported accurately and fully, in accordance with EPA GLP (40 CFR Part 160). A draft report will be provided for review by the Study Sponsor prior to study completion.

XIII. Data and Sample Retention

PROTOCOL (cont.)

- The study report and corresponding data sheets will be held in the archives of Microchem Laboratory for at least 2 years after the date of the final report and then may be destroyed. Microchem will notify Study Sponsor before any orchived informotion is destroyed. If the study is used by the Study Sponsor in support of a label claim, documentotion may be returned to the Study Sponsor for archiving at Study Sponsor's expense.
- The test substance moy be returned to the Study Sponsor at Study Spansor's request and expense within 30 days of study completion. If the Study Sponsor does not request return of the sample, it will be destroyed >90 days after study completion.

XIV. Quality Control

- The study is conducted in accordance with Microchem Laboratory's Quality Manogement System ond will undergo a full quality assurance review. All protocol amendments will be fully recorded and reported, as well as any deviations from the protocol.

XV. References

- "ASTM, International, 2013. E2871-13: Standard Test Method for Evaluating Disinfectant Efficacy against *Pseudomonas aeruginoso* Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor"
- ASTM, International, 2012. E2562-12: Standord Test Method for Quantification of *Pseudomonas aeruginasa* Biofilm Grown in CDC Reactor using Single Tube Method"
- EPA Stondard Operating Procedure MB-19; Growing a Biofilm using the CDC Biofilm Reactor.
- EPA Standard Operating Procedure MB-20; Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm.
- EPA Product Performance Test Guidelines OCSPP 810.2000 General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing, February 2018

XVI. Protocol Approval

"I, the Study Sponsor, have read and understand the study protocol. By signing this protocol I am certifying that the information and parameters accurately describe the test(s) to be completed in accordance with Good Laboratory Practice Standards (GLPS) stipulated by 40 CFR 160. I have also read, understand and agree to the terms ond conditions listed in the protocol."

Study Sponsor/Representative Signature Approving Protocol

Sponsor, Sterilex Corporation	March 21, 2018 Date
Study Director, Microchem Laboratory, LLC	22MAR2018 Date